Studies on the Intracellular Trafficking of PAMAM Dendrimer

P.S. Lai*, M.J. Shieh*, C.L. Pai*, C.Y. Wang* and P.J. Lou**

* Institute of Biomedical Engineering, National Taiwan University,
Taipei, Taiwan, f89548003@ntu.edu.tw
** Department of Otolaryngology, National Taiwan University Hospital,
Taipei, Taiwan, pjlou@ha.mc.ntu.edu.tw

ABSTRACT

Polyamidoamine (PAMAM) dendrimers are a new class of highly branched spherical polymers and have emerged as a novel synthetic drug and gene carrier. The aim of this study is to elucidate the intracellular trafficking of generation 4 (G4) PAMAM dendrimers. We conjugated G4 PAMAM to fluorescein isothiocyanate (FITC) and implemented organelle-selective dyes for double-labeling confocal microscopy studies. The results revealed that the dendrimers were endocytosed into the endo-lysosomal compartments. Certain amounts of the dendrimers shuttled between lysosomes and mitochondria at later time points. Small amounts of dendrimers were localized in the Golgi apparatus. There was no endoplasmic reticulum (ER) or nuclear localization of the dendrimers. These results not only provide us invaluable information in dendrimer biology but also have great impact to the future development of more precise dendrimer-drug or -gene delivery systems.

Keywords: nanotechnology, dendrimer, intracellular trafficking

1 INTRODUCTION

Polyamidoamine (PAMAM) dendrimers are potential vectors that can be easily modified. The dendrimer/DNA complexes will form on the basis of electrostatic interactions between the protonated amino groups of the PAMAM dendrimer and negatively charged phosphate groups of the nucleic acids. PAMAM-DNA complex has been reported for efficient transfection of a broad range of eukaryotic cells and cell lines with minimal cytotoxicity [1]. Gene transfection by PAMAM dendrimers offers significant advantages over classical transfection methods such as cationic liposomes and polylysines [2-3]. Instead of fusion with the plasma membrane, it is hypothesized that cationic lipids deliver nucleic acids into the cell predominately via an endocytotic pathway. It is proposed that PAMAM dendrimers enter the cytoplasmic compartment via the endocytotic pathway similar to that of cationic lipid. However, the fate of the dendrimer-nucleotide complexes in a biological environment and the mechanism of dendrimer-mediated cellular uptake of oligonucleotides have not been clarified. Moreover, the subcellular distribution of PAMAM dendrimers has never been deliberately studied.

2 MATERIALS AND METHODS

2.1 Preparation of FITC-Labeled PAMAM Dendrimer (G4 PAMAM-FITC)

G4 PAMAM dendrimer (50 mg, 3.51 μmole) was dissolved in DMSO (2 mL) and sodium phosphate buffer (0.1 M, pH 9, 5 mL). Fluorescein isothiocyanate (16.4 mg, 42.12 μmole) was subsequently added under nitrogen. Reaction products were ultrafiltered, and then characterized by ^{1}H NMR and atomic force microscopy (AFM).

2.2 Cytotoxicity Assay

HaCaT cells were plated into 6-well plates (10^5 cells/well) and were cultivated overnight. The cells were then incubated with G4 PAMAM, G4 PAMAM-FITC, or vehicle, respectively, for 48 h in DMEM. After the incubation, the cells were rinsed twice with phosphate-buffered saline (PBS), followed by addition of 200 μl 0.25% trypsin for 2 min. 800 μl culture medium was added to each well, mixed thoroughly, then 20μl cell suspension was dropped onto the parafilm. 20μl of 0.4% (w/v) Trypan blue was added to the cell suspension, mixed well, and scored under phase contrast microscope. Dead cells took up the blue stain of trypan blue, whereas the live cell excluded the dye. All data were obtained from at least 3 independent experiments.

2.3 Flow Cytometry

10^5 cells were seeded onto 35mm dishes, incubated with G4 PAMAM-FITC, washed with PBS, typsinized, and then transferred to centrifuge tubes. The cells were fixed with 10% formalin for 15 min on ice, and then washed twice with PBS. The pellet was resuspended in PBS and analyzed immediately by flow cytometry. Dendrimer binding/uptake was monitored using a Becton Diskinson FACS Calibur. FITC fluorescence (488-nm excitation) was determined by using a 530-nm band pass filter (30-nm band width).

2.4 Subcellular Distribution of G4 PAMAM Dendrimer

Cells were seeded on coverslips in 35mm dishes. After 18 h, the medium was replaced with G4 PAMAM-FITC (27μg) in 2 ml complete medium for 1 h (pulse), washed twice with PBS, chased for different time points, washed with PBS, and then proceeded for organelle labeling.

For lysosome (Lyso), mitochondrion (Mito), endoplasmic reticulum (ER) and nucleus staining, the cells were incubated with pre-warmed LysoTracker® Red DND-99, MitoTracker® Red CM-H_2XROS, or ER-Tracker™ Blue-White DPX (Molecular probes, USA) probe-containing medium at 37℃ for 1h. After thorough wash, the cells were fixed with formalin at room temperature for 10 min and then washed again with PBS. Nuclear counter-staining was performed by incubating the cells in the Hoechst 33342 (Molecular probes, USA) labeling solution at room temperature for 30 minutes. Then, the cells were washed and mounted for subsequent confocal fluorescence microscopy (Leica TCS SP2 Spectral Confocal System).

For Golgi staining, the cells were rinsed in HBSS/HEPES medium, and then incubated with 5μM BODIPY® TR C5-ceramide-BSA (Molecular probes, USA) in HBSS/HEPES for 30 min at 4℃. The cells were then washed twice with ice-cold medium and incubated in fresh medium at 37℃ for a further 30 min. After the incubation, the cells were washed three times with PBS, fixed with 10% formalin, and then mounted for confocal fluorescence microscopy studies.

3 RESULTS AND DISCUSSION

3.1 AFM Investigation of FITC Labeled PAMAM Dendrimer

Tapping mode AFM images of G4 PAMAM and G4 PAMAM-FITC dendrimers by spreading on a mica surface with spin-coating procedure to form films was shown in Fig. 1. The AFM image of G4 dendrimers (film deposited from the 0.01% (w/w) concentration) showed a uniform and flat surface. Tomalia et al. reported the theoretical size of the individual G4 PAMAM dendrimer is about 4 nm [4]. However, the diameters of the G4 PAMAM dendrimers, from our study, ranged between 15 to 30 nm (Fig. 1a), which is bigger than the theoretical diameter proposed by Tomalia et al. Similar results were also observed by Li et al. [5] and we believe this is due to the aggregation of G4 PAMAM dendrimers.

On the other hand, the AFM image of G4 PAMAM-FITC dendrimers (film deposited from the same concentration) showed a slight increase of the dendrimer diameters (between 20 to 40nm) with more unclear boundaries (Fig. 1b). This is because the conjugation of FITC decreases the surface charge density of PAMAM dendrimer and also the electrical attraction between PAMAM dendrimer (positive charge) and mica surface (negative charge). Thus, the dendrimer branches can relatively easily interpenetrate each other dendrimer molecules, establish intermolecular interactions and cause plate and more flat like films.

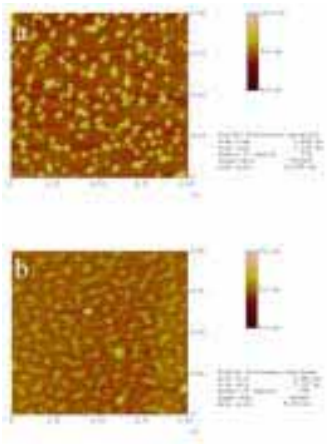

Figure 1: Tapping mode AFM images of a) G4 and b) G4 PAMAM -FITC dendrimers on mica surface.

3.2 The Cytotoxicity of G4 and G4 PAMAM-FITC Dendrimers on HaCaT Cells

The effects of G4 PAMAM and G4 PAMAM-FITC dendrimers on the viability of HaCaT cells were studied. As shown in Fig.2, G4 PAMAM and G4 PAMAM-FITC dendrimer had no conspicuous toxic effects on HaCaT cells after 48h of incubation, which means the FITC conjugation does not cause additional cell toxicity on HaCaT cells. Thus, the G4 PAMAM-FITC is reasonably designed as a probe for monitoring dendrimer uptake.

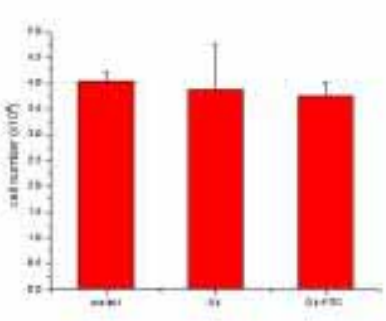

Figure 2: Effects of G4 PAMAM and G4 PAMAM-FITC dendrimers on the viability of HaCaT cells.

3.3 Cellular Uptake of G4 PAMAM-FITC

To quantify the uptake of G4 PAMAM-FITC by HaCaT cells, we carried out the flow cytometry studies. Cellular

content of the G4 PAMAM-FITC was determined by the fluorescence intensity of FITC after incubating with HaCaT cells at different time points (Fig. 3). Flow cytometry results confirmed the observation of confocal microscopy (data not shown) that a time-dependent increase of G4 PAMAM-FITC uptake by HaCaT cells. After 48h of incubation, there was a 19.7-fold increase of the fluorescence intensity as compared with that of 30 min incubation.

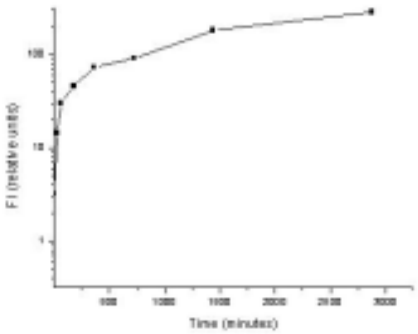

Figure 3: Flow cytometry study of G4 PAMAM-FITC uptake by HaCaT cells.

3.4 Time-Course Study of Intracellular Trafficking of G4 PAMAM-FITC

Although it is accepted that cellular uptake of polycations occurs by endocytosis, little is known about the intracellular distribution and trafficking of PAMAM dendrimers. In this study, the subcellular localization of G4 PAMAM-FITC was examined by dual-staining with fluorescent organelle probes. Fig. 4 showed the results of pulse-chase study of G4 PAMAM-FITC in HaCaT cells. The representative merged images of G4 PAMAM-FITC and organelle-specific (lysosome, mitochondria, and Golgi apparatus) dyes were shown in Fig. 4-$_{1\sim21}$. Co-localization of G4 PAMAM-FITC with the respective cytoplasmic organelle-selective dye is indicated by yellow color. Blue fluorescence ER probe was chosen because there is no other commercialized ER-Tracker™ available. The confocal micrographs of G4 PAMAM-FITC and ER probe were shown in Fig. 4-$_{22\sim28}$.

It seems that G4 PAMAM-FITC adhered to the cell membrane surface via electrostatic interaction. At 30min chase, the fluorescence of G4 PAMAM-FITC was not observed in the cytosol (Fig. 4-1, 4-8, 4-15 and 4-22). The fluorescence signal of G4 PAMAM-FITC was vaguely visualized without co-localization with any organelle probe at 1h chase, which stands for the beginning of endocytosis (Fig. 4-2, 4-9, 4-16 and 4-23). The fluorescence signal was partially co-localized with LysoTracker® after the movement of G4 PAMAM-FITC from endosome to lysosome at 3h chase. A significant co-localization of G4 PAMAM-FITC and LysoTracker® can be observed between 6h and 12h chase (Fig. 4-4, 4-5), which means more G4 PAMAM-FITC were processed into the lysosomes. Intriguingly, at 24h chase, the co-localization of G4 PAMAM-FITC and LysoTracker® decreased (Fig. 4-6) while some co-localization of G4 PAMAM-FITC and MitoTracker® was observed (Fig. 4-13). It seems that the G4 PAMAM-FITC was released from the lysosome and translocated to the mitochondria at this time point. At 48h chase, most of the G4 PAMAM-FITC entered the lysosomes (Fig. 4-7). However, there was still some co-localization of G4 PAMAM-FITC and MitoTracker® at this time point. In addition, some faint fluorescence signal of G4 PAMAM-FITC was observed in the Golgi apparatus. The fluorescence topographic profiles recorded after cells dual-labeled with G4 PAMAM-FITC and ER probe revealed no co-localization at all time points. Consistent with our prior observation, the fluorescence distribution of G4 PAMAM-FITC was pure cytoplasmic with no obvious fluorescence in the nucleus.

According to our results, G4 PAMAM-FITC adhered to the cell membrane surface at 30min, became visible within the cells at 1h, began to enter the lysosome at 3h, accumulated and increased in the lysosome from 6h to 12h, partially translocated to mitochondrial at 24h, and then accumulated in the lysosome at 48h.

It has been demonstrated that the dendrimer/DNA complex uptake is proceeded via the non-specific endocytic pathway [6]. The electrostatic interactions between the dendrimer/DNA complex and cell membrane were very important for high efficiency transfection. It has been postulated that branched cationic polymer has a high buffer capacity, owing to protonable amine group, and that enables dendrimer to act as a weak base and possesses resistance by acidification within the endosome/lysosome. The pH reduction within endosome/lysosome might lead the cationic polymer to swell, thus disrupting the membrane barrier of the organelle and promoting DNA or complex release to the cytosol. This model of "proton sponge effect" for cationic polymer was proposed by Kichler et al. [7]. However, the intracellular fate of PAMAM dendrimer alone may be a different story from the dendrimer/DNA complex. We observed that G4 PAMAM-FITC was endocytosed via the classical endosome/lysosome pathway. Strikingly, small amount of the dendrimers shuttled between mitochondria and lysosomes at later time points. Based on the current understanding of the intracellular fate of PAMAM dnedrimers, the release of G4 PAMAM-FITC from lysosome to the cytosol might occur by the osmotic swelling (the "proton sponge effect" hypothesis). However, the mechanism by which G4 PAMAM-FITC shuttled between lysosomes and mitochondria remains unclear. Further investigations are in process at our lab to clarify this issue.

Co-localization of G4 PAMAM-FITC and Golgi probe was also observed in this study that may be due to the intimate relations between the endosome, lysosome, and Golgi apparatus. It is known that the endocytosed materials

will meet the hydrolases, which are delivered from the Golgi apparatus, in the late endosomes. It is proposed that mature lysosomes form from the late endosomes by a gradual maturation process. During this process endosomal membrane proteins are selectively retrieved from the developing lysosome by transport vesicles that deliver these proteins back to endosome or the trans Golgi network (TGN). Therefore, G4 PAMAM-FITC was first endocytosed and initially delivered in vesicles to early endosomes, then passed on into late endosomes, where G4 PAMAM-FITC met hydrolases delivered from Golgi apparatus. The late endosomes then fused with lysosomes, and the transport vesicles delivered proteins and G4 PAMAM-FITC back to endosome or the TGN. This may explain the fluorescence signals of G4 PAMAM-FITC observed in the Golgi apparatus.

It is reported that the G5 dendrimer/oligonucleotides complex will enter the nuclei without dissociation [8]. In contrast, the results of this study demonstrated that G4 PAMAM-FITC can not enter the cell nuclei. Therefore, the entry of the PAMAM dendrimer into the nuclei might occur under the situation when the dendrimers were associated with DNA. On the contrary, as reported by Godbey et al., the endocytosed polyethylenimine (PEI), whether administered with or without DNA, undergoes nuclear localization [9]. The intracellular fate of another nanoparticle, polymeric micelle, is totally different from the cationic polymers. Savic et al. demonstrated the subcellular distribution of polymeric micelle with major localization in mitochondria and Golgi apparatus after 24h incubation [10]. In addition to the mitochondria and Golgi apparatus, the micelles could also be observed in the lysosome and ER. These differences may be due to the different types of nanomaterials. However, the different intracellular fates between these two cationic polymers, PAMAM and PEI, are very interesting. Further studies about the effects of DNA on the nuclear translocation of dendrimers are still undergoing at our lab.

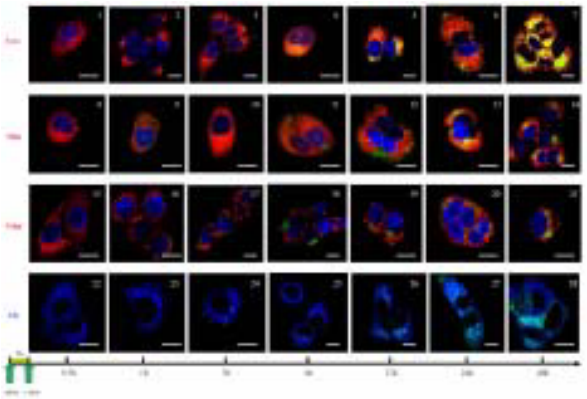

Figure 4: Intracellular trafficking of G4 PAMAM-FITC dendrimer in HaCaT cells

4 CONCLUSION

Interest in the surface engineering of nanomaterials has increased significantly in recent years. The nanomaterial surface can be modified for targeting, imaging and drug or gene delivery to develop the multi-functional nanodevice. The purpose of this study is to elucidate the subcellular distribution and intracellular trafficking of PAMAM dendrimer. We have, for the first time, delineated the detailed subcellular distribution and intracellular trafficking of PAMAM dendrimer using time-course studies. The most striking finding is that although G4 PAMAM-FITC was endocytosed following the endosome/lysosome pathway, certain amounts of the dendrimers shuttled between lysosomes and mitochondria at later time points. Small amount of the dendrimers was observed in the Golgi apparatus, also at later time points, but there was no ER or nuclear localization of the dendrimers. The results of this study not only provide us invaluable information in dendrimer biology but also have great impact to the future development of more precise dendrimer-drug or -gene delivery systems.

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